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The goal of this project is to learn the structure and assembly characteristics of a family of secretory proteins (SP's). SP's are synthesized in salivary glands of aquatic larvae of the midge, Chironomus. Larvae construct underwater feeding and pupation tubes using insoluble silk-like fibers composed of one or more SP's. During the past year two objectives were met. We completed the identification of another SP gene. Using cDNA and anti-(synthetic peptide) antibody probes we demonstrated that Balbiani ring 3 on polytene chromosome IV contains a gene that encodes a 6 kb poly(A)$^+$ mRNA for a 185-kDal SP (sp185). This gene belongs to a class of SP genes that are expressed throughout the 4th instar. sp185 contains a cluster of 3 Cys every 22 residues. An assembly assay developed under this contract was used to study disassembly and reassembly of SP complexes in vitro. A purified fraction of 1000-kDal SP's was able to assemble into fibrous complexes which, on the basis of electron microscopy and quantitative circular dichroism spectroscopy, were nearly identical to complexes formed by unfractionated SP's.

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SECRETORY POLYPEPTIDES ENCODED BY BALBIANI RING GENES

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Project Summary

The aim of this project is to learn about the structure, developmentally regulated synthesis and assembly of a family of secretory proteins (SPs) into an insoluble polymer of silk-like threads. SPs are exclusively synthesized in salivary glands of aquatic larvae of the Dipteran, Chironomus. All SPs studied to date are composed of tandemly repeated amino acid sequences. Recombinant cDNA probes are used to map SP-coding genes on polytene chromosomes, identify their mRNAs on Northern blots and derive the amino acid sequence of their encoded protein. cDNA probes and anti-SP antibodies are used to study the level at which SP gene regulation occurs during larval development and under conditions of galactose-induced alterations in gene expression. SP assembly in vitro is being studied by a combination of physical, electron microscopic and biochemical methods. We hope to learn which SPs interact with each other and what is the chemical nature of these interactions. We eventually plan to determine the spatial distribution of SPs within assembled complexes by making three-dimensional tomographic reconstructions from immunoelectron micrographs. This experimental system provides a unique opportunity to study how naturally occurring soluble proteins can assemble into an insoluble fiber that functions in an aqueous environment.

Results from the Prior Year

Identification and mapping of an sp185 gene. A randomly primed cDNA clone was shown to hybridize in situ to Balbiani ring 3 (BR3) on salivary gland polytene chromosome IV. Northern blots indicated that the cDNA originated from a 6-kb poly(A)$^+$ RNA. The cDNA insert contains 483 nucleotides which, in contrast to other SP genes studied thus far, does not contain tandemly repeated sequences. The mRNA-like strand contains one open reading frame of 160 amino acids. The encoded polypeptide contains a periodic distribution of cysteine [Cys-X-Cys-X-Cys-X_6-Cys] about every 22 residues. An 18-residue portion of this sequence was selected for solid phase chemical peptide synthesis. Affinity purified antipeptide antibodies were used to identify the BR3 gene product as a 185-kDa secretory protein (sp185).

Developmental studies showed that sp185 and its mRNA were present in salivary glands throughout the 4th larval instar. Thus

sp185 and a previously studied family of 1000-kDa SPs (spIs) are encoded by a larval class class of genes that are expressed throughout the 4th instar. This contrasts with the developmentally regulated expression of the sp140 and sp195 genes (previously referred to as sp130 and sp180, respectively) which is maximal during prepupal stages of larval development. These results enhance the notion that secretory threads assembled in vivo may be dynamic at least in part due to compositional changes in SPs.

In vitro assembly/disassembly of SPs. We began in vitro studies of the mechanism of assembly of SPs into macromolecular complexes, the structure of assembled SPs and the contribution of individual SPs to the assembled structure. These studies are based on an in vitro assembly assay that was developed under this contract. From measurements of solution turbidity and electron micrographs, we observed that SPs were isolated from the lumen of salivary glands as complexes. These complexes most likely represent initial stages of assembly. Denaturation and reduction of disulfide bonds disrupted the complexes, and removal of denaturing and reducing agents resulted in reassembly of complexes. The circular dichroic spectrum of the complexes indicated that the assembled proteins had the tertiary structure $\alpha+\beta$. Purified spIs (the 1000-kDa SPs) were shown to assemble into complexes with both similar morphology, using electron microscopy, and a similar dichroic spectrum to that of unfractionated native complexes. We estimated from quantitative dichroism measurements that spIs were approximately 15% α-helix, 28-30% β-sheet, 26-28% β-turn and 25% other secondary structure. Our data provide the first experimental evidence in support of the notion that spIs form the fibrous backbone of larval silk.

Plans for Next Year

SP-coding genes. We will continue to identify and map additional SP-coding genes using recombinant DNA procedures which have been successful to date. The next probe on our agenda is a cDNA which hybridizes to a 4.8-kb salivary gland RNA. Its 3-kb insert is being sequenced and we plan to make antipeptide antibodies to identify its protein product. To facilitate this endeavor, we have arranged to exchange hybridization and antibody probes between two other labs that study SP gene expression in related species of Chironomus. Professor J.-E. Edstrom (Lund, Sweden) sent putative SP cDNA clones from C. pallidivittatus that were selected in expression vectors using anti-SP antibodies. I have arranged a visit to Professor I.I. Kiknadze (Novosibirsk, U.S.S.R.) to initiate an interspecific comparison of SP gene structure between C. tentans and C. thummi. Each lab has gene probes that the other lacks.

We have abandoned earlier plans to obtain additional sequence data for sp140 and sp195 since Lars Wieslander's lab (Stockholm, Sweden) will soon publish full-length cDNA and genomic sequences for these genes.

Structural organization of SP complexes. We will continue to study SP-SP interactions now focusing on spIs. These SPs are comprised of alternating "constant" and "subrepeat" domains. We have synthesized milligram quantities of constant and subrepeat peptides. Their structure and assembly characteristics will be compared to native spIs purified by glycerol gradient centrifugation. We hope to identify one or more domains responsible for in vitro assembly.

We will continue to pursue further fractionation of SPs. We eventually wish to purify sp185 to learn about the status of its multiple Cys residues (free -SH groups, disulfide bonds, metal coordination?). Concurrently we need to develope a scheme that will enable us to purify sp140 and sp195 (prepupal SPs) to test whether their addition to purified spIs in vitro results in assembly of structurally modified SP complexes.

Finally we hope to initiate the microscopic identification of SPs in complexes assembled in vitro and in vivo. Anti-peptide antibodies will be used on partially fractionated SPs and unfractionated complexes to see if we can identify and localize specific proteins. These pilot studies will set the stage for determining the spatial distribution SPs within a complex. To attain this goal, however, we will require mouse monoclonal antibodies that we plan to raise this year. These antibodies will be indispensable reagents for our future immunoelectron microscopic studies, particularly those involving electron microscope tomography.